CHAPTER 7

Aristolochic Acid Nephropathy: An Environmental and Iatrogenic Disease

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Contents

The *Aristolochiaceaea* family of herbaceous plants, specifically members of the genus *Aristolochia,* has been used for medicinal purposes for more than 2500 years [1]. Remarkably, the extensive *materia medica* describing the therapeutic use of *Aristolochia* rarely mentions intrinsic toxicity. Recently, aristolochic acid (AA), a principal component of all *Aristolochia* sp., was shown to be the toxic principle responsible for the syndromes known as Chinese herb nephropathy (CHN) and endemic (Balkan) nephropathy (EN). Both disorders are associated with a high incidence of urothelial (transitional cell) cancer and appear to constitute a single disease entity, best designated as aristolochic acid nephropathy (AAN).

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Advances in Molecular Toxicology, Volume 3
ISSN 1872-0854, DOI: 10.1016/S1872-0854(09)00007-1

The epidemiology and pathophysiology of CHN and EN have been reviewed extensively [2–7] and the association of these diseases with human cancer is the subject of several comprehensive reports [8,9]. Guided by the hypothesis that AA is the common etiologic agent in CHN and EN [10,11], we review the molecular and clinical toxicology of this powerful nephrotoxin-carcinogen. Additionally, based on the traditional use of *Aristolochia* in herbal remedies, we posit that AAN represents a long-overlooked iatrogenic disease and an international public health problem of considerable magnitude [12].

1. Toxicity of Aristolochic Acid (AA)

Toxicologists have long been aware of the toxic properties of *Aristolochia* sp. In 1825, while investigating the homeopathic principle that ''like cures like,'' Jörg performed experiments on himself and his colleagues, revealing the acute toxic properties of *Aristolochia serpentaria* [13]. Among the effects experienced after ingesting multiple doses of the root (2.5–7.5 gm, estimated to contain ~5 mg AA) were intense gastrointestinal symptoms and polyuria. Later, Orfila, the founder of modern quantitative toxicology, demonstrated the lethal effects of *Aristolochia clematitis* in short-term experiments performed on dogs [14]. More than a century ago, Pohl administered the extracts prepared from the seeds and roots of *A. clematitis* to rabbits, revealing the nephrotoxic effects of the partially purified phytotoxin [15]. Dumić [16] and Martinčiić [17] reported that horses ingesting hay contaminated with *A. clematitis* developed chronic renal failure and described the histopathology of the diseased kidneys [17]. Subsequently, Mengs documented the acute and chronic toxicities of purified AA in rodents, including its carcinogenicity [18–20]. The molecular and cellular mechanisms underlying AA cytotoxicity have been explored in cultured renal proximal tubule cells and in rodent models of AAN (reviewed in [21]).

2. Chemistry of AA

Aristolochia herbs contain a number of structurally related nitrophenanthrene carboxylic acids, principally aristolochic acid I (AA-l) and aristolochic acid II (AA-ll) [22]. Many of the studies discussed here utilize the naturally occurring mixture of AA-l and AA-ll designated for purposes of this discussion as AA.

3. Chinese Herb Nephropathy (CHN)

Between 1990 and 1992, ~1800 otherwise healthy Belgian women inadvertently ingested *Aristolochia fangchi* in conjunction with their participation in a weight-loss regimen. Several of these women developed rapidly progressive renal interstitial fibrosis leading to chronic renal failure [23]. Histopathologic examination revealed a corticomedullary gradient of interstitial fibrosis, and marked atrophy of proximal tubules with glomeruli generally being spared. Approximately 5% of women ingesting the herbal supplement developed end-stage renal disease (ESRD) ultimately requiring dialysis or renal transplantation [3]. When the diseased kidneys were removed, urothelial cell atypia and carcinoma were found in the upper urinary tract [24]. Specifically, among 39 patients undergoing prophylactic bilateral nephrectomy, 46% had urothelial cancers located primarily in the renal pelvis and upper ureter [25]. All but two of the remaining patients displayed mild-to-moderate urothelial cell dysplasia.

Definitive evidence that AA was responsible for CHN was obtained when aristolactam (AL)-DNA adducts were detected in renal tissues (reviewed in [26]). Additionally, Cosyns *et al.* reproduced the nephrotoxic and carcinogenic effects observed in humans by treating rabbits with low doses of AA [27]. Based on these reports, the term aristolochic acid nephropathy (AAN) was suggested to reflect the etiologic role of this phytotoxin in CHN [28].

The dramatic revelation that AA is a powerful nephrotoxin and carcinogen for humans drew attention to the worldwide distribution and use of *Aristolochia* sp. as herbal remedies. Not surprisingly, subsequent reports (reviewed in [9]) described almost 200 patients outside of Belgium in whom chronic renal failure followed ingestion of *Aristolochia* herbs.

Undoubtedly, published reports represent only a small fraction of AAN cases. In support of this assertion, we note that in a single year in China, 320,000 kg of *Aristolochia manchuriensis* was harvested for medicinal purposes [29]. And, in 2003, 5000 kg of Qing Mu Xiang and 10,000 kg of Tian Xian Teng, *Aristolochia* herbals listed in the 2005 Chinese Pharmacopeia, were produced in the Shanghai district alone. Thus, despite heavy and widespread use of Aristolochic herbals in China, very few cases of AAN were reported prior to 1999 [30].

Epidemiologic studies in Taiwan, where the prevalence of chronic kidney disease (CKD) is among the highest in the world, reveal a strong association between CKD and the general use of herbal therapies [3l]. Too, a survey of prescriptions filled between1997 and 2003 in Taiwan revealed that one-third of the population had been treated with herbal products known to contain or suspected to be contaminated with *Aristolochia* [32].

CKD also is recognized as a major public health problem in India [33]. Traditional Ayurvedic medicines used in India and elsewhere include several species of *Aristolochia*, predominantly *Aristolochia indica* and *Aristolochia bracteolata*. Vanherweghem [34] has suggested that the nephrotoxicity associated with *Aristolochia* herbal remedies may contribute to the high incidence (27.8%) of chronic interstitial nephritis in India [35].

Thus, the failure to relate the traditional uses of *Aristolochia* sp. to its delayed nephrotoxic and carcinogenic effects has created a global health problem that will require the attention of public health authorities for the foreseeable future [13].

4. Endemic (Balkan) Nephropathy (EN)

EN is a chronic tubulointerstitial disease occurring in rural villages located near tributaries of the river Danube in Bosnia and Herzogovina, Bulgaria, Croatia, Romania, and Serbia. First described 50 years ago [36–39], EN is estimated to affect at least 25,000 individuals in countries harboring this disease, while another 75,000 men and women residing in endemic regions are believed to be at risk [40]. Significant epidemiologic features of EN include its focal occurrence in certain farming villages; a familial but not inherited pattern of disease, frequently affecting members of the same household; occurrence only in individuals older than 18 years of age; presence in less than 10% of households in endemic villages; and a strong association with upper urinary tract transitional cell (urothelial) cancer (UUC) [4–6,41].

Until quite recently, the etiology of EN remained obscure [42]. A variety of environmental agents, including mycotoxins, heavy metals, viruses, and trace-element deficiencies have been investigated as potential causative agents [42,43], with ochratoxin A (OTA), being a principal focus of EN research in recent years. Residents of villages in endemic areas are exposed to relatively high concentrations of OTA; however, elevated serum levels of OTA have been detected in residents of countries throughout the world [44] while EN is limited to isolated villages in Balkan countries [4–6]. In fact, OTA has never been associated directly with nephrotoxicity in humans [45] and a comprehensive review of the literature concluded that published epidemiologic studies are inadequate to assess a cause-and-effect relationship between OTA and human cancer [46]. Thus, the weight of scientific evidence, including the inability of OTA to form DNA adducts under physiologic conditions [47,48], argues strongly against OTA or other mycotoxins playing a role in the etiology of EN [10,21].

Current experimental evidence strongly supports the view [49–51] that chronic dietary poisoning by AA accounts for all of the important

characteristics of EN, including the geographical distribution, characteristic pattern of renal tubulointerstitial fibrosis, and increased risk of developing UUC. Earlier, Kazantzis [52] and Ivić [53] suggested that *A. clematitis* might contain the etiologic agent responsible for EN; however, this insightful observation was not pursued. Our impetus to investigate the role of AA in EN was prompted by the striking clinical and histopathologic similarities between EN and the nephropathy observed in the cluster of Belgian women [54].

5. AAN = BEN = CHN

In the following section, we review critically the toxicology of AA, focusing on renal proximal tubules and urothelial cells, which are targeted by the toxin. In so doing, we integrate observations on the pathophysiology of CHN and EN with research on animal models of AAN and mechanistic studies of the molecular and cellular effects of AA (reviewed in [21]).

6. Dose–Toxicity Relationship

Estimates have been made of the amount of AA ingested by ~100 Belgian women who developed end-stage renal failure [3,55] and by many of the ~200 sporadic cases of CHN reported in the world literature (reviewed in [9]). Our analysis of the dose–toxicity relationship assumes that AA-induced DNA damage in cells is cumulative and largely irreversible. Thus, 1800 Belgian women received a maximum of 0.025 mg/kg of AA over a period averaging 13 months, with 5% of this group developing end-stage renal failure 3–85 months after administration was stopped [3]. Similarly, Chinese patients estimated to have ingested 0.7–1. 5 mg AA per day intermittently over 1–10 years in the form of pills containing *A. manchuriensis* developed chronic renal failure, sometimes within 6 months of initial exposure to the herb ([9] and X. Li, unpublished data). Higher doses of AA, such as those employed in a Phase I clinical study of AA (1 mg/kg for 3 or more days) [56], and in some Chinese patients (10 mg/day for 10 days) [57] resulted in acute renal toxicity.

In EN, 20 years or more may elapse before renal function becomes clinically apparent. The slow progression of this endemic disease likely reflects a low level of exposure to AA. Thus, Croatian farm families who ingest bread contaminated by AA are estimated to accumulate over 8–10 years the same amount of AA as did Belgium women with CHN in a single year [49]. The number of residents in Croatian endemic areas who depend on home-baked bread has decreased substantially in the past 20 years (Jelaković *et al.*, unpublished data). Decreased dietary exposure to AA is

expected to translate into a lower incidence of EN and a delayed age of onset of nephrotoxicity. The observed shift to the older age group among newly diagnosed EN cases and among EN patients beginning dialysis [5] is in line with this prediction.

7. Renal Histopathology

AAN is marked by progressive tubulointerstitial fibrosis resulting in ESRD. Comparison of the renal histopathology of patients with CHN and EN revealed a striking feature, namely, a corticomedullary gradient of interstitial fibrosis present in both diseases [54]. This gradient, reported only in AAN and cadmium-induced nephropathy [58] is even more pronounced in renal biopsies obtained prior to ESRD.

New Zealand White rabbits treated over a period of 17–21 months with low doses of AA developed impaired renal tubular function and interstitial fibrosis with a corticomedullary gradient [27]. Following treatment with AA, C3H/He mice developed acute tubular necrosis and interstitial fibrosis while C57BL/6 mice are resistant to similar doses of the toxin [59]. AA-I and AA-II exhibit similar genotoxic potential [60], however, AA-I is solely responsible for the observed nephrotoxicity in C3H mice [59,60]. Histopathologic findings in Wistar rats include necrosis of outer medullary proximal tubules and urothelial dysplasia. As in mice, renal injury in rats is manifested initially by an acute inflammatory/necrotic phase lasting several days, followed by a chronic phase, consisting of interstitial inflammation, tubular atrophy, and interstitial fibrosis [61]. Biochemical markers were consistent with epithelial–mesenchymal transition, though an alternative mechanism involving TGF-β activation of fibroblasts may account for some of the interstitial fibrosis involved [62].

8. Proximal Tubular Function

AAN is characterized by proximal tubule dysfunction, including persistent glycosuria, aminoaciduria, and low-molecular weight (tubular) proteinuria (LMWP) [3,4]. Normally, LMWP are filtered at the glomerulus, reabsorbed by endocytosis in the proximal tubule, and catabolized. Proximal tubule damage interferes with this process; thus, the urinary LMWP/albumin ratio is strikingly elevated in patients with CHN and EN compared with patients with glomerular disease [63]. This parameter provides a useful biomarker for detecting early proximal tubule dysfunction in AAN [64–66].

Rabbits given AA intraperitoneally 5 days a week for 17–21 months developed impaired proximal tubule function manifested by glycosuria and

tubular proteinuria [27]. In Wistar rats, high doses of AA disrupted proximal tubule function, as reflected by proteinuria, glycosuria, and neutral amino acid enzymuria [67]. Both animal models reproduce the main features of AAN in humans. The rat was used to investigate early functional impairment of the proximal tubule, revealing that the outer medullary S3 segment of the proximal tubule was the preferential target of AA [67].

9. Urothelial (Transitional) Cell Cancer

The strong association between exposure to AA and the development of urothelial neoplasia was first recognized in Belgian women with CHN [24,68]. Ultimately, 40–45% of this group developed multifocal high-grade transitional cell carcinomas located primarily in the upper urinary tract [25]. Based on this observation, prophylactic bilateral nephroureterectomy has been strongly recommended for CHN and EN patients requiring dialysis or renal transplantation [69].

UUC also are prevalent among residents of endemic villages in the Balkan region. In the former Yugoslavia, data from more than 2000 cases of UUC were used to explore the epidemiology of this uncommon cancer [70]. The prevalence of UUC in certain endemic regions can be as much as 100-fold greater than in the country as a whole [71]. More than two-thirds of these cancers are localized in the renal pelvis or ureter [72,73] compared to ~5% of UUC among patients residing in nonendemic areas [74]. In Serbia, the fraction of urothelial cancers located in the renal pelvis and ureter increased from 18% in the period 1921–1952 to 63% in 1953–1960 [70,72].

Patients undergoing surgery for UUC often exhibit signs of renal dysfunction. Petković *et al.* recorded "renal failure" in 63% (111/187) of patients with UUC [75]. This important observation was confirmed by Nikolić in a review of 710 patients with UUC, 65% of whom had creatinine clearances of less than 80 ml/min [70]. Recently, 9 of 11 residents of endemic villages undergoing nephroureterectomy for UUC were found to have histopathologic changes in the renal cortex compatible with EN [50]. Thus, UUC may precede, accompany, or follow manifestations of nephrotoxicity in patients with EN. The age at which patients in endemic regions develop symptoms associated with UUC has increased steadily over the past 30 years [76] again suggesting that environmental exposure to AA is diminishing over time.

10. AL-DNA Adducts

Both AA-I and AA-II are subject to enzymatic nitroreduction in mammalian cells, generating active nitrenium intermediates (Fig. 1) that react with the exocyclic amino groups of deoxyadenine and deoxyguanine

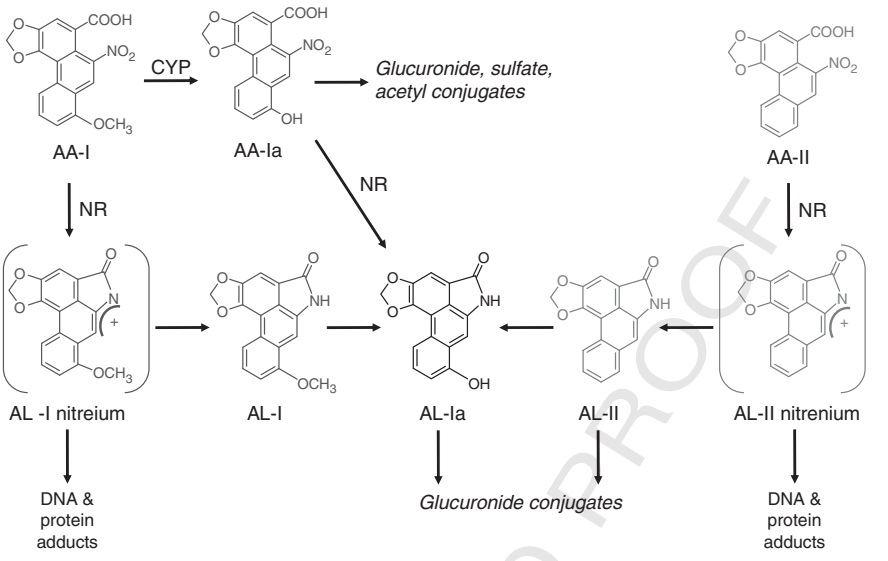

Figure 1 Principal urinary metabolites of aristolochic acids (AA) in rat. Cytochrome P450 (CYP) catalyzed demethylation of AA-I generates AA-Ia, which is subject, in turn, to Phase II biotransformation to form glucuronide, sulfate, and acetyl conjugates. Enzymatic nitroreduction (NR) of AA-I or AA-II yields the biologically inactive aristolactams AL-I and AL-II. Reactive nitrenium intermediates form covalent adducts with DNA and proteins.

bases of DNA (reviewed in [26]). Both AL-l- and AL-ll-derived DNA adducts have been detected in the renal cortex of patients with BEN and/or UUC [50] and in persons ingesting botanical products containing AA (reviewed in [9]).

The level of AL-DNA adducts in target tissues represent the integration of external exposure with interindividual variability in metabolism, nucleotide excision repair, and other cellular processes, providing a tangible link between exposure to the genotoxin and its cytotoxic and carcinogenic effects [77]. Their usefulness as biomarkers is enhanced by their persistence in target tissues. For example, in humans, AL-DNA adducts were detected 36–89 months following termination of exposure to AA [26,50]. Thus, among Belgian women, AL-DNA adduct levels ranged from 0.1 to 17 adducts per 10^8 deoxynucleotides (dNs), with dA-AL-I being the major adduct found in the renal cortex while dA-AL-II and dG-AL-I were present in significantly lower amounts [26].

In patients with EN, adduct levels ranged from 8 to 59 adducts per 10^8 dNs for dA-AL-l and 2–62 adducts per 10^8 dNs for dG-AL-l [50]. In urothelial tumors, adduct levels were significantly lower, 0.7–1.6 and

0.3–0.5 adducts per 10^8 dNs for dA-AL and dG-AL, respectively [50]. In rodent models of AAN, AL-DNA adducts accumulate in target tissues [78]; however, the prominent cytotoxic effects of AA-I in proximal tubule cells appear unrelated to its DNA-damaging effects [60].

11. Mutational Signature of AA

Mutational analysis of the p53 tumor suppressor gene in UUC tumors from patients residing in endemic villages revealed that the frequency of A:T → T:A mutations was 78% [50]. In contrast, A:T → T:A mutations occur infrequently in transitional cell carcinomas of the renal pelvis (0%), ureter (5%), and bladder (4.8%) in patients from nonendemic regions [79]. Importantly, the p53 mutation pattern in UUC associated with EN is consistent with the mutational spectra induced by AA-I (or by a mixture of AA-I and AA-II) in (i) the H-ras gene of rats [80]; (ii) transgenic rodent models [81]; (iii) the p53 gene in a urothelial cancer from a patient with CHN [82]; (iv) site-specific mutagenesis studies in which a single dA-AL adduct is transfected into NER-deficient human cells (Yang and Moriya, unpublished data); and (v) immortalized (Hupki) mouse cell lines carrying the human p53 gene [83]. Indeed, the predominance of A:T → T:A transversions in the p53 mutational spectrum is widely recognized as a mutational signature for human exposure to AA [10,50,83,84].

12. Genetic Considerations

Only 5% of the 1800 Belgian women exposed to similar amounts of AA developed AAN [3] and the reported prevalence of EN is 3–7% [5]. Similarly, various strains of mice exhibit differing susceptibilities to the toxic effects of AA [59,60]. Thus, genetically determined interindividual variability could contribute to the risk of developing AAN and/or its associated UUC [85]. Rarely, however, does a single mutated gene result in a high absolute risk of the associated cancer or other disease. Thus, susceptibility to AA most likely is influenced by the additive or synergistic effects of several genes, none of which individually may result in a large phenotypic effect. Furthermore, the cumulative effect of susceptibility to this environmental mutagen may result from complex interactions among multiple genes and from gene–environment interactions [86]. Molecular epidemiologic studies have been undertaken to potentially capture such interactive effects in EN, and mouse models of AAN have been used to identify genes controlling susceptibility to the nephrotoxic effects of AA [87].

Polymorphisms that affect susceptibility to AA are likely to be found in genes responsible for its absorption, bioactivation, detoxification, excretion, and/or transport. Metabolism of AA, mediated by enzymes known collectively as xenobiotic metabolizing enzymes (XMEs), has been demonstrated in humans and animals treated with AA [88] (Fig. 1). *O*-demethylation, mediated by Cytochrome P450s, results in rapid detoxification of AA-1 in rodents [89]. Thus, functional polymorphisms in XMEs may affect the level of critical intermediates produced during biotransformation, some of which bind to DNA and proteins. Similarly, polymorphisms in DNA repair enzymes can affect an individual's ability to excise the AL-DNA adducts that initiate UUC [90]. Although individual risks associated with such polymorphisms might be low, they have potentially great public health relevance (i.e., population-attributable risk) because of their high population frequency [91].

13. AAN as an Iatrogenic Disease

The varied uses of *Aristolochia* (birthwort) have been traced from the fourth century BC to the eighteenth century AD, based on excerpts from the original literature [1]. In a more specific approach, the actual cumulative doses of *Aristolochia* employed in treating illness during the Greco-Roman period were estimated by Scarborough who suggested that this early usage likely resulted in unrecognized iatrogenic disease as a result of the herb's intrinsic toxicity [92].

Extending this novel strategy, we have analyzed the use of *Aristolochia* herbal combinations in Ayurvedic and Chinese medicine. Our immediate goal is to document clearly the past and present use, worldwide, of *Aristolochia*, a family of herbs recently shown to be nephrotoxic and carcinogenic to humans.

The first description of *Aristolochia* is found in ''Enquiry into Plants'' by Theophrastus (300 BC) who described the morphology of the plant and its medical uses [94]. At that time, *Aristolochia* was used for snakebites, as a general antidote against poisons, to clean wounds, to alleviate insomnia, cure uterine malfunctions, ease bowel obstruction, and relieve symptoms of dropsy (edema). In the Hippocratic *Diseases of Women,* anonymous writers of that era discuss the use of *Aristolochia* as an oxytocic agent to aid women in childbirth and abortifacient, obstetric and gynecologic practices that continue to this day [95].

Arguably the most influential medical work of classical antiquity, *De Materia Medica,* written ca. 70 AD by Dioscorides, includes almost all herbs then employed in Greco-Roman pharmacology. Individual chapters are devoted to *Aristolochia rotunda, Aristolochia longa, and Aristolochia clematitis* [96]. Interestingly, not one word of caution was expressed regarding the potential toxicity of these herbs [92].

The influence of *De Materia Medica* on Western and Islamic medicine was profound. Dioscorides' descriptions of *Aristolochia* were translated into Arabic and Latin and this information was preserved throughout the Byzantine period and the Middle Ages [93]. With the advent of the printing press, information originating in *De Materia Medica* was incorporated (often verbatim) into the printed herbals of the Renaissance, which enjoyed wide distribution in Europe [97]. Ultimately, an entry for *Aristolochia* appeared in the *Pharmacopeia Londinesis of 1618* and; following that, in other official compendia and textbooks of pharmacology. Literature discussing properties of birthwort continued into the twentieth century [98]. Once again, usage of this herb was not associated with nephrotoxicity or cancer.

Ayurverda, one of several codified systems of traditional medicine, enjoys the same legal status in India as does allopathic medicine. The *Carakasamhita*, dating from ca. 400 AD and constituting one of the earliest Ayurvedic medical treatises, contains descriptions of 341 plant products. Scholars have identified in this classic text several species of *Aristolochia* used in Ayurveda today. Thus, patients currently treated by Ayurvedic practioners are exposed to the toxic effects of these native herbs.

Gandhanakuli, *isvari*, and *nakuli,* identified as *Aristolochia indica* Linn *or A. bracteolata* Lam are mentioned in the *Carakasamhita* as antidotes to snakebite and for intermittent fevers (G.J. Meulenbeld, personal communication). Similar applications of *Aristolochia* sp. appear in more recent Indian pharmacopeia where the herb continues to be used for children with fever as well as an emmenagogue and antiarthritic [99].

In China, the earliest record of herbal medicines is the Shen Nong Ben Cao Jing (Shennong Herbal), compiled sometime between the first century BC and the second century AD [93]. Three hundred sixty-five agents were listed in this text and are divided into superior, medium, and inferior based on their relative toxicity [103]. Mou Dou Ling (fruit of *Aristolochcha* debilis) Au3 first appears in Lei Gong Pao Jiu Lun (ca. 421 AD) with no mention of its toxic effects.

The Ben Cao Gang Mu, compiled by Li Shi-Zhen in the latter part of the sixteenth century, was based on the author's experience and on data obtained from earlier herbals. This Chinese herbal classic describes 1892 "drugs" (with 1110 drawings) including many species of *Aristolochia.* For 400 years, the Ben Cao Gang Mu remained the principal source of information in traditional Chinese medicine and the work was translated into numerous languages, reflecting its influence in countries other than China.

In the mid-twentieth century, Ben Cao Gang Mu was replaced by modern *materia medica*, the most comprehensive source being Zhong Hua Ben Cao (Encyclopedia of Chinese Materia Medica), published in 1999 [100]. The Encyclopedia lists 23 species of *Aristolochia*, again with little mention of toxicity. The Chinese government currently lists the following *Aristolochia* herbs: *A. manshuriensis* (stems), *A. fangchi* (root), *A. debilis* (root

and fruit), and A. contorta (*fruit*), two of which (Mou Dou Ling and Quingmuxiang) appear in the 2005 Pharmacopeia.

For thousands of years, herbs have been used for medicinal purposes and in the twenty-first century 80% of the world's population still rely on herbal remedies to treat symptoms of disease. *Aristolochia* herbals play an important role in this tradition. The governments of China and India, countries that each have populations in excess of one billion, have been slow in implementing measures to reduce human exposure to this toxic herb. Thus, from a global perspective, we conclude that AAN and its associated upper urothelial cancer continues to exist as it has in centuries past as a ''silent'' but omnipresent iatrogenic disease.

ACKNOWLEDGMENT

The authors express their appreciation to Professor John Scarborough, Professor Gerrit Jan Meulenbeld and Ms. Jie Wang, respectively, for their translations of ancient Greek, Sanscrit and Chinese text. We thank Dr. Jovan Nikolic for his critique of the section on upper urinary tract cancer and Ms Annette Oestreicher for her skilled editorial work. The National Institute of Environmental Health Sciences (grant ES-04068), Fogarty International Center (grant 1203–07042) and Croatian Ministry of Science supported our collaborative research on aristolochic acid nephropathy.

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